STUDIES ON THE CONFORMATIONAL CHANGES OF METALLOPROTEINS INDUCED BY ELECTRONS IN WATER–ETHYLENE GLYCOL SOLUTIONS AT LOW TEMPERATURES. CYTOCHROME C.

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1. Introduction

A new conception of the physical mechanism of enzymatic reactions was formulated in [1,2]. An essential feature of the conception is the assumption that the chemical changes of the active centre of an enzyme (substrate binding as a rule) take place much more faster than the following conformational change of the macromolecule, which is a relaxation by its nature. If the conception is valid, it should be possible to detect intermediate unequilibrium states appearing during the catalytic act where the modified active centre of the enzyme is surrounded by the polypeptide chain with largely unchanged secondary and/or tertiary structures.

Indeed, it was shown [3,4] by means of pulse radiolysis of cytochrome *c*–water solutions that the change of optical characteristics of the active centre involves fast and slow stages: substantial alterations of the electron structure proceed during the first 10^{-5} sec after a pulse and a small additional adjustment of the spectral characteristics necessary to achieve the complete coincidence with the reduced cytochrome *c* spectrum requires $10^{-2} - 10^{-1}$ sec. Taking into account the crystallographic data [5] the authors consider the slow stage as reflecting the influence of the macromolecular conformational transition on the reduced state of the active centre, thus supporting in fact our prediction.

We tried to separate local modifications of the active centre induced by reduction of the metal ions from the following macromolecular conformational transitions carrying out the reduction of diluted protein solutions at 77°K. At the temperature of liquid nitrogen essential changes of the shape and dimensions of macromolecules are kinetically forbidden, because the corresponding substantial alterations of large solvent regions cannot take place and a macromolecule is fixed in an unequilibrium state with changed active centre and unchanged bulk of the globule. Cytochrome *c* from horse heart and cytochrome of the type *c* from *Chorella* were chosen as the first objects for the investigations.

2. Experimental

Horse heart cytochrome *c* obtained from 'Koch & Light Lab.', 'Reanal' (Hungary) and 'Cracow' (Poland) was purified by gel filtration. The cytochrome of the type *c* from *Chlorella* was a gift from Dr. A. A. Mutuskin. Chemical reduction of the proteins at room temperature was conducted with a solution of Na-ascorbate (pH 7). Reduction of the proteins at the low temperature was carried out in water–ethyleneglycol rigid glass-like mixtures (50% of ethylene glycol) by electrons produced by γ-irradiation (^{60}Co), dose range 3–10 M rad. The absorption spectra were recorded always at 77°K on a 'Specord UV-VIS' spectrophotometer, samples placed in a cell for turbid media. Before recording the spectra after γ-irradiation the samples were illuminated with a 400 W incandescent lamp till the complete vanishing of the absorption band of trapped electrons.

Protein concentration in water–ethylene glycol solution was as a rule 10^{-4} M, for measurements in Soret region – 10^{-5} M.

3. Results and discussion

The low temperature spectrum of the cytochrome *c* reduced by ascorbate at room temperature did not change after γ-irradiation. It means that at doses used in our investigation we can neglect the spectral changes due to radiational damages of the protein. (The same is true in the case of cytochrome of the type *c* from *Chorella*).

On the other hand, irradiation of the oxidized cytochrome caused substantial spectral changes (see fig. 1). Judging by the character of the changes the active centre of the cytochrome *c* was reduced during the irradiation. Indeed, two bands in the green region of the spectrum with characteristic distribution of intensity indicate the presence of a low-spin ferrous

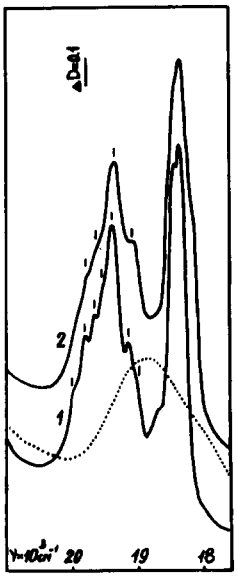

Fig. 1. Absorption spectra of horse heart cytochrome *c* in water–ethylene glycol mixture (50:50) at 77°K. Concentration of the protein in preparation 10^{-4} M (for measurements in Soret region 10^{-5} M). The spectrum of the oxidized cytochrome (. . . . .); The spectrum of the cytochrome reduced at room temperature (1); The spectrum of the cytochrome reduced at 77°K (2). The curves are shifted arbitrary along the ordinate axis.

porphyrin compound. However, the spectrum of the cytochrome *c* reduced at the low temperature (LRC) differs distinctly from the spectrum of the chemically reduced cytochrome (CRC) (see fig. 1). The Soret band of the CRC peaks at 414.5 nm whereas the maximum of the same band of the LRC is located at 416 nm (the mean values of the measurements). The α-band of the CRC consists of three components with maxima at 547 nm – α_1-component, 545 nm – α_2-component and 537 nm – α_3-component, but the three components of the α-band of the LRC are located at $\lambda\alpha_1^1$ = 550 nm, $\lambda\alpha_2^1$ = 547 nm and $\lambda\alpha_3^1$ = 545 nm. Instead of the seven countable components in the β-band of the CRC ($\lambda\beta_1$ = 526 nm, $\lambda\beta_2$ = 524 nm, $\lambda\beta_3$ = 518 nm, $\lambda\beta_4$ = 513 nm, $\lambda\beta_5$ = 511 nm, $\lambda\beta_6$ = 507 nm and $\lambda\beta_7$ = 501 nm) we see only four distinguishable peaks in the case of the LRC ($\lambda\beta_1^1$ = 524 nm, $\lambda\beta_2^1$ = 518 nm, $\lambda\beta_3^1$ = 513 nm and $\lambda\beta_4^1$ = 507 nm). It is reasonable to assume that the differences in the spectra reflect differences in the geometry of the active centres and in the locations of amino acid residues in the vicinity of the porphyrin rings. The differences observed by us had appeared evidently because conformational transition induced by the change of the Fe-porphyrin redox state could not take place in the rigid water–ethylene glycol solution, and we were able to see the reduced active centre in the 'oxidized' macromolecule.

The interpretation tnat the observed differences indicate to the differences in the geometry of the active centres and in their nearest environments is supported by our observations that the character of the differences depends on the origin of the protein and on the composition and pH of the solutions used. We have investigated several cytochromes in different solutions (manuscripts in preparation) and sometimes the unequilibrium states appeared had had rather unusual spectral characteristics. For example, in the case of cytochrome of the type *c* from *Chlorella* (fig. 2) besides the differences in the intensities of the components of the β-band, a 6 nm red shift of the α_1-component ($\lambda\alpha_1$ = 552 nm in the CRC and $\lambda\alpha_1^1$ = 558 nm in the LRC respectively) was observed.

Heating of the samples reduced at the low temperature up to the room temperature results in the complete disappearance of the observed differences. (Instead of curves 1.1 and 2.1. curves 1.2. and 2.2. were recorded after the heating.) Thus we were able

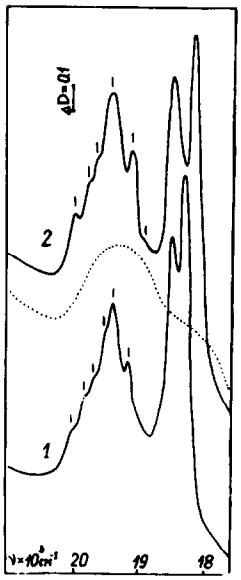

Fig. 2. Absorption spectra of the cytochrome of the type *c* from *Chlorella*. Conditions and curves as in fig. 1.

to observe the predicted relaxation in the both cases as well.

Last, not least, qualitative coincidence of the results of the works [3,4] and ours – substantial alternations of the spectrum of cytochrome proceeded during the fast stage of reduction in pulse radiolysis method and immediately after the reduction of the active centre without conformational changes in our low temperature studies – serves as an essential evidence of the relaxational character of the conformational changes in water solution at room temperature. In this case as in ours the modifications of the active centre and its nearest environment take place without large conformational changes and a conformational relaxation is needed to establish the complete thermodynamic equilibrium state of the molecule. It should be noted here that the state observed by us was not necessarlily the same registered in [3] or [4] after the fast stages had passed (10–20 sec after a pulse). Indeed, relaxation pathways of macromolecules in these studies might be different due to drastic differences in the state of solution – liquid water in the case of pulse radiolysis method [3,4] and a solid water–ethylene glycol glass in our case.

Acknowledgement

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